Molecular Mechanisms of Transformation of C3H/10T1/2 C1 8 Mouse Embryo Cells and Diploid Human Fibroblasts by Carcinogenic Metal Compounds

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Carcinogenic arsenic, nickel, and chromium compounds induced morphological and neoplastic transformation but no mutation to ouabain resistance in 10T1/2 mouse embryo cells; lead chromate also did not induce mutation to ouabain or 6-thioguanine resistance in Chinese hamster ovary cells. The mechanism of metal-induced morphological transformation was likely not due to the specific base substitution mutations measured in ouabain resistance mutation assays, and for lead chromate, likely not due to this type of base substitution mutation or to frameshift mutations. Preliminary data indicate increases in steady-state levels of c-*myc* RNA in arsenic-, nickel-, and chromium-transformed cell lines. We also showed that carcinogenic nickel, chromium, and arsenic compounds and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induced stable anchorage independence (AI) in diploid human fibroblasts (DHF) but no focus formation or immortality. Nickel subsulfide and lead chromate induced AI but not mutation to 6-thioguanine resistance. The mechanism of induction of AI by metal salts in DHF was likely not by the type of base substitution or frameshift mutations measured in these assays. MNNG induced AI, mutation to 6-thioguanine resistance, and mutation to ouabain resistance,and might induce AI by base substitution or frameshift mutations. Dexamethasone, aspirin, and salicylic acid inhibited nickel subsulfide, MNNG, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced AI in DHF, suggesting that arachidonic acid metabolism and oxygen radical generation play a role in induction of AI. We propose that nickel compounds stimulate arachidonic acid metabolism, consequent oxygen radical generation, and oxygen radical attack upon DNA. Intracellular reduction of Cr(VI) to Cr(V) or other species that generate oxygen radicals leads to Cr(V) or oxygen radical attack upon DNA. Arsenite causes chromosome breaks. We propose that arsenic, nickel, and chromium compounds then cause small deletions or mutations in the 5' or 3' regulatory regions of the c-*myc* and other protooncogenes, resulting in stabilization of c-*myc* RNA and higher steady-state levels of c-*myc* RNA and protein. We also postulate that nickel-induced oxygen radical generation, Cr(V) ions or oxygen radicals generated by chromium, and arsenite induce inactivating mutations or deletions in tumor suppressor genes. Arsenic, nickel, or chromium compound-induced neoplastic transformation is postulated to proceed through a combination of activation of c-*myc* and/or other protooncogenes and inactivation of tumor suppressor. — Environ Health Perspect 102(Suppl 3):119–125 (1994).

Key words: morphological and anchorage-independent transformation, carcinogenic metal salts, prostaglandins, oncogenes

Introduction

Occupational exposure of humans to arsenic, nickel, or chromium compounds correlates with increased frequencies of skin, lung, esophageal, and nasal carcinomas (*1–12*), and nickel and insoluble hexavalent chromium compounds are carcinogenic when administered to animals (*1–16*). Epidemiological evidence indicates that occupational exposure to arsenic compounds correlates with increased skin and respiratory cancer in humans, but this contrasts with the lack of carcinogenicity of arsenic compounds in animals, presenting an apparent paradox (*1,3,6–8,12*). These studies have been extended to the cellular level, demonstrating that nickel, hexavalent chromium, and arsenic compounds induce (*17–24*) or promote (*25*) morphological transformation of cultured rodent cells and induce anchorage independence in diploid human fibroblasts (*7–10,26,27*).

Significant effort has gone into studying the molecular mechanisms of metal carcinogenesis, and the molecular basis of metal carcinogenesis is beginning to be understood. Metal compounds induce chromosomal aberrations in cultured mammalian cells (*28,29*); however, except for hexavalent chromium compounds and platinum coordination complexes, carcinogenic metal compounds are inactive or at best weakly active in bacterial and mammalian cell mutagenesis assays (*7,8,30*). The *in vitro* mammalian and bacterial mutagenesis assays currently available do not effectively detect the DNA alterations caused by carcinogenic metal salts (*28,29*). In this manuscript, I review recent studies from my laboratory on induction of morphological transformation in C3H/10T1/2 Cl 8 (10Tl/2) mouse embryo cells and anchorage independence in diploid human fibroblasts by carcinogenic metal salts and hypothesize the possible mechanisms of metal salt-induced cell transformation.

Materials and Methods

C3H/10Tl/2 Cl 8 mouse embryo fibroblasts were cultured according to the meth-

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ods of Reznikoff et al. (*31*). Assays were conducted to quantitate chemically induced cytotoxicity and morphological transformation (*23,24,32,33,34*). Assays to detect chemically induced mutation to ouabain resistance in 10Tl/2 cells were performed as previously reported (*23,24,33*). Diploid human fibroblasts were derived from circumcised human foreskins and cultured according to the methods of Biedermann and Landolph (*26,27*). Assays to detect metal-induced cytotoxicity, anchorage independence, and mutation to 6-thioguanine resistance and ouabain resistance were also as described by Biedermann and Landolph (*26,27*).

Results

Induction of Morphological Transformation of 10T1/2 Cells by Carcinogenic Arsenic, Nickel, and Chromium Compounds

10T1/2 cells are an aneuploid, immortal mouse cell line derived from embryos of C3H mice that are contact inhibited, nontumorigenic, and have a low frequency of spontaneous transformation (*31*). These cells exhibit a high frequency of morphological transformation when treated with chemical carcinogens or radiation (*7,8,23,24,32–35*). They are also a useful model cell culture system used to study the mechanisms of chemically induced neoplastic transformation and to detect carcinogens by their ability to induce morphological transformation of these cells (*7,8,34,35*).

We have been studying the ability of carcinogenic metal salts to induce morphological transformation of 10T1/2 cells to gain insight into the mechanisms of metal carcinogenesis. Our cumulative studies indicate that carcinogenic arsenic, nickel, and chromium compounds induce morphological transformation of 10T1/2 cells (*7,8*).

We first examined the ability of 10T1/2 cells to phagocytose carcinogenic insoluble nickel compounds. We found that 10T1/2 cells readily phagocytose nickel subsulfide particles, and the phagocytosed particles can be readily seen in phagocytic vesicles (Figure 1). The phagocytosis of nickel subsulfide and crystalline nickel monosulfide by 10T1/2 cells occurs when low concentrations of these compounds (0–50 μM) are added to the medium (Figure 2*A*) (*24*). Over the concentration range that phagocytic uptake of nickel compounds occurs, we also observed a concentration dependent cytotoxicity in 10T1/2 cells treated with the insoluble nickel compounds nickel subsulfide and nickel monosulfide (Figure 2*B*), With nickel oxide. we did not observe discrete phagocytic vesicles, but nevertheless, we observed cytotoxicity in 10T1/2 cells treated with from 0 to 1 mM nickel oxide (Figure 2*B*) (*24*).

We next studied the ability of carcinogenic nickel compounds to induce morphological transformation in 10T1/2 cells (*24*). The insoluble carcinogenic nickel compounds nickel subsulfide, nickel monosulfide, and nickel oxide (greenish preparation) all induced dose-dependent transformation in 10T1/2 cells, over the same concentration ranges that were phagocytosed and induced cytotoxicity (Table 1). This indicated that phagocytosis of particulate nickel compounds is an initial step in the processes of cytotoxicity and cell transformation in 10T1/2 cells (*24*). Similar observations have been made by Costa and co-workers in Syrian hamster cells (*17,18*). Nickel subsulfide induced primarily type II foci, and cell lines derived from these foci did not grow in soft agarose (Table 2). Nickel monosulfide-induced largely type II and occasionally type III foci, and three nickel monosulfide induced type II and type III foci gave rise to cell lines that grew in soft agarose (*24*). Nickel oxide induced type II and occasionally type III foci, and one of these type II and type III foci gave rise to a cell line that grew in soft agarose and formed fibrosarcomas in nude mice (*24*).

Conversely, the soluble nickel compounds nickel sulfate and nickel chloride, which are noncarcinogenic or substantially less carcinogenic than the insoluble nickel compounds, did not induce morphological transformation of 10T1/2 cells (*7,8,24*). The inability of soluble nickel compounds to induce cell transformation is consistent with whole animal carcinogenicity studies (*1,2,4,5,7–10,13–16*). This indicates that results for induction of morphological transformation in 10T1/2 cells with nickel compounds correlates with the results of whole animal carcinogenesis assays using nickel compounds. Our cell transformation responses with nickel compounds are therefore specific responses.

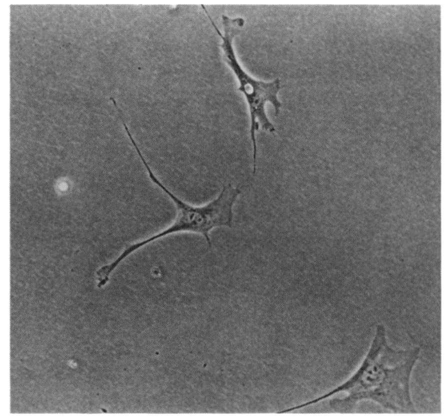

Figure 1. Phagocytosis of particles of nickel subsulfide by logarithmically growing 10T1/2 cells. Reprinted from Miura et al. (*24*) with permission.

We previously developed an assay detecting chemically induced (*33*) stable (*36*), specific (*37*) base substitution mutations (*38*) to ouabain resistance that results from a mutation in a gene encoding (Na,K)-ATPase activity on murine chromosome 3 (*39*) and confers a ouabain-

Table 1. Morphological transformation of 10T1/2 cells by nickel subsulfide and nickel oxide and lack of mutation to ouabain resistance.

Treatment	Survival, %	Total type II and type III foci per 20 dishes	Ouabain-resistant mutants/ one million survivors
DMSO	100	0	1
Nickel subsulfide			
0.5 μM	85	3.4	1
1.0 μM	91	2.2	1
5.0 μM	85	3.1	1
10.0 μM	70	7.9	1
20.0 μM	50	4.1	1
40.0 μM	26	6.4	1
Nickel oxide			
50.0 μM	81	0.5	ND
100.0 μM	61	0.8	ND
200.0 μM	61	1.4	ND
400.0 μM	47	3.5	1
600.0 μM	39	6.2	1
800.0 μM	26	4.9	1
1000.0 μM	02	4.7	1

ND not determined. These data are reprinted in modified form from Miura et al. (*24*), with permission.

Table 2. Summary of expression and structure of the c-*myc* protooncogene in metal transformed 10T1/2 mouse embryo cell lines.

Type of transformed 10T1/2 cell line	Fraction of transformed 10T1/2 cell lines with: Higher steady state levels of c-*myc* RNA	Amplification of the c-*myc* gene	Rearrangement of the c-*myc* gene
Four sodium arsenite-transformed 10T12/cell lines[a]	3/4	0/4	0/4
Four nickel oxide and nickel monosulfide-transformed 10TI/2 cell lines[b]	1/4	0/4	0/4
Two lead chromate transformed 10TI/2 cell lines[c]	2/2	0/4	0/4

[a]Data from JR Lillehaug, D Evans, and JR Landolph, (unpublished data). [b]Data from T Sakuramoto, D Evans, T Miura, and JR Landolph, (unpublished data). [c]Data from M Dews and JR Landolph, (unpublished data).

resistant (Na,K)-ATPase activity (*35,40*). We next studied the ability of nickel compounds to induce mutations in this assay. Concentrations of nickel subsulfide and nickel oxide that induced cytotoxicity and morphological transformation in 10T1/2 cells did not induce base substitution mutations to ouabain resistance in 10T1/2 cells (Table 1) (*24*). This indicates nickel compounds likely do not induce morphological transformation by inducing the specific, restricted type of base substitution mutations that are detected in an assay for mutation to ouabain resistance (*24*).

Recent preliminary studies from our laboratory indicate that nickel monosulfide- and nickel oxide-transformed 10T1/2 cell lines frequently express higher steady-state levels of c-*myc* RNA (Table 2). We hypothesize that transformation of 10T1/2 cells by nickel compounds results in small deletions or small mutations in the 3' or 5' regulatory regions of the c-*myc* and c-H-*ras* protooncogenes, leading to activation of these protooncogenes via this mechanism and hence a stabilization of the protooncogene RNAs and the increased steady-state levels and increased half-lives of these mRNAs that we observe. Studies to test this hypothesis critically in our laboratory are in progress (T Sakuramoto, D Evans, T Miura, S Boone, and JR Landolph, unpublished data).

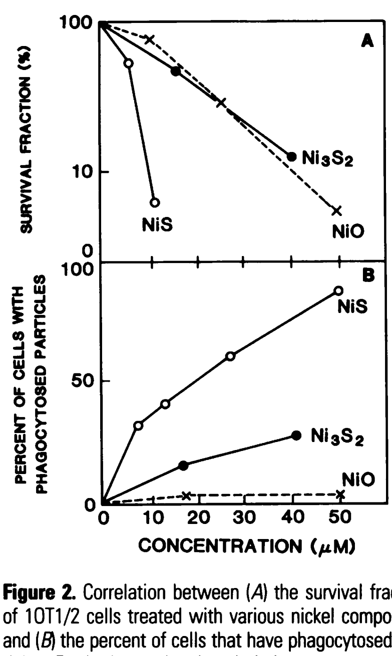

Figure 2. Correlation between (*A*) the survival fraction of 10T1/2 cells treated with various nickel compounds and (*B*) the percent of cells that have phagocytosed particles. For both panels, closed circles represent nickel subsulfide-treated cells, open circles represent crystalline nickel monosulfide-treated cells, and x represents nickel oxide-treated cells. Reprinted from Miura et al. (*24*), with permission.

We were also able to show that lead chromate induced a low but dose-dependent and reproducible frequency of type III morphological transformation (*23*), the strongest type of transformation in 10T1/2 cells (*32,34*). The transformed cells stably maintained a focus-forming phenotype, grew in soft agarose, and formed fibrosarcomas when injected into nude mice (*23*). Lead chromate-treated cells had a large number of vacuoles and extruded cytoplasm over the particles of lead chromate, apparently in an attempt to phagocytose lead chromate particles (*23*). Calcium chromate, potassium dichromate, and strontium chromate did not induce morphological transformation in 10T1/2 cells (*23*). Strontium chromate was slightly soluble in culture and eventually dissolved and calcium chromate did not induce transformation even when added to the cells as a particulate in acetone suspension (*23*). This indicated that unique physicochemical properties of insoluble lead chromate particles were responsible for its uptake, likely by phagocytosis, and its ability to induce cytotoxicity and cell transformation (*23*), and correlated with earlier observations that the slightly soluble hexavalent chromium compounds are also carcinogenic in animal bioassays (*4,5,7,8*).

We showed that lead chromate induced morphological transformation in 10T1/2 cells but not mutation to ouabain resistance in 10T1/2 or CHO cells nor mutation to 6-thioguanine resistance in CHO cells(*23*). In addition, calcium chromate did not induce morphological transformation in 10T1/2 cells or mutation to ouabain resistance in 10T1/2 or CHO cells but did induce mutation to 6-thioguanine resistance in CHO cells (*23*). Hence, we speculated that lead chromate induced morphological transformation by a mechanism not involving the specific type of base substitution mutations or frameshift mutations detectable in assays for mutation to ouabain resistance or 6-thioguanine resistance. Most recently, we obtained preliminary evidence that in two lead chromate-transformed 10T1/2 cell lines, the steady-state levels of c-*myc* RNA are 6- to 8-fold higher, and the half-life of c-*myc* RNA is increased in these cell lines (M Dews and JR Landolph, unpublished data). Our current working hypothesis is that lead chromate induces mutations or small deletions in the 5' or 3' regulatory regions of the c-*myc* gene, leading to a c-*myc* RNA with an increased half-life. This may be due either to deletions or mutations generated by the reduction of Cr(VI), leading to either Cr(V) or oxygen radicals generated by intracellular reduction of Cr(VI).

Third, we observed that sodium arsenite induced a low but reproducible yield of morphological transformation in 10Tl/2 cells(JR Landolph and C Troesch, unpublished data). Cloning of arsenite- transformed cell lines yielded cell lines that formed type II and type III foci, grew in soft agarose, and formed fibrosarcomas in nude mice. We also found that sodium arsenite promoted cell transformation initiated by 3-methylcholanthrene. Pentavalent sodium arsenate and potassium arsenate did not induce morphological transformation, indicating that this transformation was specific for the trivalent state of arsenic (JR Landolph and CT Troesch, unpublished data). These data are consistent with epidemiological studies indicating that arsenic compounds are carcinogenic to humans (*1–3,6,8,12*). A resolution to the apparent paradox of the noncarcinogenicity of arsenic in animal bioassays could be that sodium arsenite is a weak cell-transforming agent and a promoting or co-carcinogenic agent that is not easily detected in the relatively insensitive animal bioassays. A sec-

Table 3. Summary of the effects of inhibitors of arachidonic acid release and metabolism on induction of anchorage independence in diploid human fibroblasts.[a]

Compound	Induction of anchorage independence	Inhibition of anchorage independence by Dexamethasone	Aspirin	Salicylic acid
Nickel subsulfide	+	+	+	+
MNNG	+	+	+	+
TPA	+	ND	+	ND

[a]Preliminary data from Biedermann KA, Nwankwo JO, Weng J, and Landolph JR, unpublished data. ND not determined.

ond possibility is that humans are more sensitive to arsenic-induced carcinogenesis than are rats and mice.

Preliminary work from our laboratory has first ruled out the hypothesis that carcinogenic metal compounds induce morphological transformation in 10T1/2 cells by inducing amplification or gross rearrangement of known protooncogenes such as *c-myc*, converting them into oncogenes (Table 2). We are now testing a second hypothesis that carcinogenic metal compounds cause mutations or small deletions in the 5' or 3' regulatory regions of specific protooncogenes, converting them into activated oncogenes. We are also testing a third hypothesis that carcinogenic metal salts cause mutational inactivation or deletion of tumor suppressor genes, thereby inactivating them. To date, we have found preliminary evidence for increased steady-state levels of *c-myc* RNA in lead chromate, nickel oxide, and sodium arsenite transformed 10T1/2 cell lines. We have not found amplification or rearrangements of the *c-myc* genes in these cell lines (T Sakuramoto, T Miura, M Dews, J Lillehaug, and JR Landolph, unpublished data). We are currently studying whether there are small mutations or deletions in the 3' or 5' regulatory regions of this gene in these transformed cell lines that might account for their activation and higher steady-state levels of the *c-myc* transcripts (Table 2).

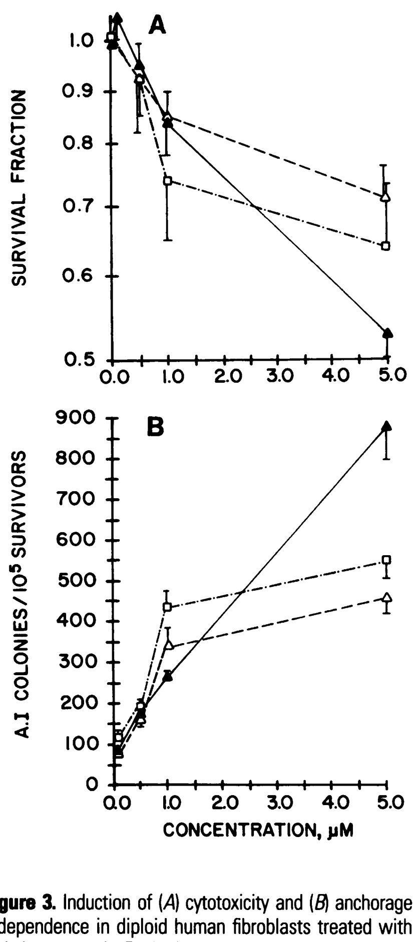

Figure 3. Induction of (*A*) cytotoxicity and (*B*) anchorage independence in diploid human fibroblasts treated with nickel compounds. For both panels. closed triangles represent nickel subsulfide-treated human fibroblasts; open squares represent nickel acetate-treated cells, and open triangles represent nickel sulfate-treated cells. Reprinted from Biedermann and Landolph (*26*) with permission.

Induction of Anchorage Independence in Diploid Human Fibroblasts

Recently, we began to study the molecular mechanisms by which carcinogenic metal salts induce transformation of cultured diploid human fibroblasts. As a source of human fibroblasts, we used circumcised human foreskins. Previous studies have shown that organic carcinogens could induce anchorage independence in cultured human fibroblasts (*41–43*). We showed that nickel compounds [nickel subsulfide, nickel acetate, and nickel subsulfide (Figure 3) (*26*), hexavalent chromium compounds (lead chromate, potassium dichromate, calcium chromate, and chromium trioxide) (*26,27*), and arsenic compounds (sodium arsenite and sodium arsenate, (*26*)] induced dose-dependent anchorage independence (AI) in diploid human fibroblasts. Metal-induced AI was a stable phenotype (*26,27*). To date, we have not observed other transformation phenotypes, such as morphological transformation. All the metal-induced anchorage-independent cell strains had saturation densities comparable to those of normal human fibroblasts, and they all eventually senesced (*26,27*). The specificity of carcinogenic metal salt-induced AI is indicated by the fact that manganese chloride, mercuric acetate, and calcium chloride, which are not carcinogenic, did not induce AI (*27*).

Nickel subsulfide did not induce mutation to ouabain resistance or to 6-thioguanine resistance (*26*) and lead chromate did not induce mutation to 6-thioguanine resistance (*27*) at concentrations that were cytotoxic, and induced AI in diploid human fibroblasts. Hence, nickel subsulfide and lead chromate likely induced anchorage independence by mutations of the type not easily measured in assays for 6-thioguanine resistance. We speculate that metal-induced oxygen radical generation and consequent radical-induced mutations might be part of the mechanism of nickel subsulfide and lead chromate-induced AI in diploid human fibroblasts. Calcium chromate, potassium dichromate, and MNNG did induce mutation to 6-thioguanine resistance and AI over the same concentration ranges, indicating that mutation might be a mechanism by which these compounds induced AI (*27*).

Recently, we have begun to test the hypothesis that stimulation of arachidonic acid metabolism and consequent generation of oxygen radicals are part of the molecular mechanism by which metal compounds induce AI in diploid human fibroblasts. We have found that nickel subsulfide-induced AI in human fibroblasts was inhibited by dexamethasone, aspirin, and nordihydroguaieretic acid. Similarly, MNNG-induced AI was also inhibited by these three inhibitors of arachidonic acid release (dexamethasone) and metabolism (aspirin, which inhibits cyclooxyenase activity) and oxygen radical persistence [salicylic acid, which scavenges oxygen radicals (*40*)]. In addition, the tumor promoter TPA induced AI only when applied to the soft agar, and the TPA-induced anchorage-independent phenotype was reversible and only manifested in the presence of TPA. However, induction of AI by TPA was also inhibited by dexamethasone and aspirin (RA Biedermann, JO Nwankwo, J Weng, and JR Landolph, unpublished data; summarized in Table 3).

Conclusions

It is very clear that the mechanisms of metal carcinogenesis are complex and only beginning to become understood, and that the mechanisms of carcinogenesis for each metal are specific to that metal (*44*). With these considerations in mind, however, a number of conclusions and interesting speculations may be drawn from the work in our laboratory on mechanisms of metal-induced cell transformation. For the carcinogenic insoluble nickel compounds such as nickel subsulfide, we (*24*) and others (*17,18*) have evidence that these compounds are phagocytosed and that large amounts of these compounds are therefore taken up into individual murine (*24*), hamster (*17,18*), and human (*26*) fibroblasts. In addition, our recent preliminary results indicate that inhibitors of arachidonic acid release and its metabolism by cyclooxygenase and an oxygen radical scavenger (salicylic acid) inhibit nickel subsulfide-induced AI in diploid human fibroblasts. We therefore speculate that nickel subsulfide induces membrane perturbations that activate the prostaglandin synthesis cascade, resulting in generation of oxygen radicals. This could explain our inability to measure mutation to ouabain resistance in 10T1/2 cells (*24*) or mutation to ouabain resistance or to 6-thioguanine resistance in diploid human fibroblasts (*26*) following treatment of these cells with nickel subsulfide, since oxygen radicals do not induce the type of mutation that is easily measured in these assays. At this time, we cannot assess the fraction of nickel-generated oxygen radicals that derive from stimulation of arachidonic acid metabolism pathway versus the fraction that derive from protein-bound nickel ions that bind to DNA and generate oxygen radicals, since it is known that nickel ions bind to proteins that can then bind to DNA (*9,45,46*).

Second, we also have preliminary evidence that there are increased steady-state levels of c-*myc* RNAs in nickel oxide and nickel monosulfide transformed 10T1/2 mouse embryo cells (T Sakuramoto, T Miura, D Evans, and JR Landolph, unpublished data). We speculate that this may result from nickel compound-generated oxygen radicals, which may induce mutations in the 3' or 5' regulatory regions of these protooncogenes, leading to enhanced stability of these RNAs and contributing to the induction and maintenance of the transformed phenotype. We know that multiple activated oncogenes may cooperate in cell transformation(*47–49*), and we are studying how many oncogenes are activated in nickel transformed cell lines.

We also hypothesize that nickel ions generate oxygen radicals, and that these oxygen radicals are responsible for inactivating tumor suppressor genes (*50*), both known tumor suppressor genes such as the retinoblastoma gene, *Rb* (*51*) and the *p53* suppressor gene (*52*), suppressor genes that are just becoming identified and understood such as the suppressor gene on human chromosome 11 (*53*), and novel suppressor genes just being discovered, such as the senescence-mediating suppressor gene that is a target for nickel-induced cell transformation discovered by Costa's group (*54*). We also speculate that nickel compounds will both inactivate genes such as the *p53* gene and also activate it to a dominantly acting negative oncogene. Further work is in progress in our laboratory and in other laboratories to test these hypotheses.

It still is unclear as to what the active ionic species are that induce chromium carcinogenesis. Chromate induces DNA cross-links in rat liver and kidney (*55*) and binds to chromatin and DNA (*56*). There is much current speculation as to whether Cr(VI) is reduced to Cr(V), which is a proximate carcinogen, or whether it is reduced to lower oxidation states, which might generate oxygen radicals (*44*). Our work with the strong carcinogen lead chromate (*57*) has given preliminary results that lead chromate transformed lOT1/2 cell lines have higher steady-state levels of c-*myc* RNA (M Dews and JR Landolph, unpublished data). We are testing the hypothesis that these higher steady-state levels of c-*myc* RNA are due to mutations in the 3' or 5' regulatory regions of the c-*myc* gene, leading to a longer half-life of c-*myc* RNA and contributing to maintenance of the transformed state. Our results with lead chromate indicate that it does not induce mutation to ouabain resistance in 10T1/2 or CHO cells or mutation to 6-thioguanine resistance in CHO cells (23). Hence, we speculate that this compound may generate Cr(V) and/or generate oxygen radicals that induce mutations or small deletions in the 3' or 5' regulatory regions of the c-*myc* gene activating it to an oncogene, by leading to an increased half-life of c-*myc* RNA. Work is in progress in our laboratory to test this hypothesis.

Finally, we have also found preliminary evidence for increased steady-state levels of c-*myc* expression in sodium arsenite transformed cell lines. There was no amplification of the c-*myc* gene in arsenite-transformed 10T1/2 cell lines (JR Lillehaug, D Evans, and JR Landolph, unpublished data). This is interesting, because Lee et al. have found that arsenite induced amplification of the dihydrofolate reductase gene in mouse 3T6 cells (*58*). Since we have found that sodium arsenite does not induce mutation to ouabain resistance in 10T1/2 cells (JR Landolph and C Troesch, unpublished data), we again postulate that arsenite induces mutations in 3' or 5' regulatory regions of the c-*myc* gene in these transformed cell lines. The precise mechanism by which arsenite induces such mutations is not clear. Work in our laboratory is currently in progress to determine the type of mutations arsenite induces in the c-*myc* gene that contributes an increased half-life of c-*myc* RNA and to maintenance of the transformed phenotype (M Dews and JR Landolph, unpublished data).

In the case of arsenic-, nickel-, and chromium-induced cell transformation, we postulate that these compounds also cause mutation and/or deletions in tumor suppressor genes that inactivate these genes. We postulate that a combination of mutation or deletion induces activation of single or multiple protooncogenes into oncogenes, plus a mutational or deletional inactivation of tumor suppressor genes, is part of the mechanism of metal carcinogenesis.

REFERENCES

1. IARC. Nickel and Certain Nickel Compounds (Group 2A) and Nickel Refining (Group 1). In IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol 1–29, Suppl 4. Lyon: International Agency for Research on Cancer, 1982;167–170.
2. IARC. Chromium and Chromium Compounds. In IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol 23. Lyon: International Agency for Research on Cancer,1980;205.
3. IARC. Some Metallic Compounds, Arsenic and Arsenic Compounds. In IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Vol 23. Lyon: International Agency for Research on Cancer, 1980.
4. Norseth T. Industrial viewpoints on cancer caused by metals as an occupational disease. In. Origins of Human Cancer, Book A: Incidence of Cancer in Humans, Vol 4. Cold Spring Harbor Conferences on Cell Proliferation (Hiatt HH, Watson J D, Winsten J. A, eds). Cold Spring Harbor, NY, 1977;159–167.
5. Hernberg S. Incidence of cancer in populations with exceptional exposure to metals. In: Origins of Human Cancer, Book A: Incidence of Cancer in Humans, Vol 4. Cold Spring Harbor Conferences on Cell Proliferation (Hiatt HH, Watson JD, Winsten JA, eds). Cold Spring Harbor, New York, 1977;147–157.
6. Peters JM, Thomas D, Falk H,

Oberdorster G, SmithTJ. Contribution of metals to respiratory cancer. Environ Health Perspect 70:71–83 (1986).
7. Landolph JR. Molecular and cellular mechanisms of transformation of C3H/lOTl/2 Cl 8 and diploid human fibroblasts by unique, carcinogenic, non-mutagenic metal compounds: a review. Biol Trace Elem Res 21:459–467 (1989).
8. Landolph JR. Neoplastic transformation of mammalian cells by carcinogenic metal compounds: cellular and molecular mechanisms. In: Biological Effects of Heavy Metals, Vol II, Metal Carcinogenesis (Foulkes EC, ed). Boca Raton, FL:CRC Press,1990;1–18.
9. Cohen M, Latta D, Coga T, Costa M. Mechanisms of metal carcinogenesis: the reaction of metals with nucleic acids. In: Biological Effects of Heavy Metals, Vol II, Metal Carcinogenesis (Foulkes EC, ed). Boca Raton, FL :CRC Press, 1990;19–76.
10. Christie NT, Katsifis SP. Nickel Carcinogenesis. In: Biological Effects of Heavy Metals, Vol II. Metal Carcinogenesis (Foulkes EC, ed). Boca Raton, FL:CRC Press, 1990;95–128.
11. Yu MC, Garabrant DA, Peters J M, Mack TM. Tobacco, alcohol, diet, and cancer of the esophagus. Cancer Res 48:3843–3849 (1988).
12. Smith AH, Hopen-hayn RC, Bates MN, Goeden HM, Hentz-Picciot I, Duggan HMN, Wood R, Kosrett MJ, Smith MT. Cancer risks from arsenic in drinking water. Environ Health Perspect 97:259–267 (1992).
13. Sunderman FW Jr. Mechanisms of nickel carcinogenesis. Scand J Work Environ Health 15:1–12 (1989).
14. Sunderman FW, Maenza RM. Comparisons of carcinogenicity of nickel compounds in rats. Res Commun Chem Pathol Pharmacol 14: 319–330 (1976).
15. Ottolenghi AD, Haseman JK, Payne WW, Falk HL, MacFarland HN. Inhalation studies of nickel subsulfide in pulmonary carcinogenesis of rats. J Natl Cancer Inst 54:1165–1172 (1974).
16. Sunderman, FW. Carcinogenic effects of metals. Fed Proc 37:40–44 (1978).
17. Heck JD, Costa M. Influence of surface and dissolution on the selective phagocytosis of potentially carcinogenic particulate metal compounds. Cancer Res 43:5652–5658 (1983).
18. Costa M, Mollenhauer H. Carcinogenic activity of particulate nickel compounds is proportional cellular uptake. Science 209: 515–517 (1980).
19. Costa M, Nye JS, Sunderman FW, Allpass PR, Gordos B. Induction of sarcomas in nude mice by implantaton of Syrian hamster embryo fetal cells exposed in vitro to nickel subsulfide. Cancer Res 39: 3591–3597 (1979).
20. DiPaolo JA, Casto B. Quantitative studies of *in vitro* morphological transformation of Syrian hamster embryo cells by inorganic metal salts. Cancer Res 39:1008–1013 (1979).
21. Saxholm H, JK, Reith A, Brogger A. Oncogenic transformation and cell lysis in C3H/lOTl/2 cells and increased sister chromatid exchange in human lymphocytes by nickel subsulfide. Cancer Res 41:4136–4139 (1981).
22. Lee TC, Oshimura M, Barrett JC. Comparison of arsenic-induced cell transformation, cytotoxicity, mutation, and cytogenetic effects in Syrian hamster embryo cells in culture. Carcinogenesis 6:1421–1426 (1985).
23. Patierno SR, Banh D, Landolph JR. Transformation of C3H/lOTl/2 mouse embryo cells to focus formation and anchorage independence by insoluble lead chromate but not soluble calcium chromate: relationship to mutagenesis and internalization of lead chromate particles. Cancer Res 48:5280–5288 (1988).
24. Miura T, Patierno SR, Sakuramoto T, Landolph JR. Morphological and neoplastic trransformation of C3H/10T1/2 mouse embryo cells by insoluble carcinogenic nickel compounds. Environ Mol Mutagen 14:65–78 (1989)
25. Rivedal E, Sanner T. Metal salts as promoters of *in vitro* morphological transformation of hamster embryo cells initiated by benzo(a)pyrene. Cancer Res 41:2950–2953 (1981).
26. Biedermann KA, Landolph JR. Induction of anchorage independence in human diploid fibroblasts by carcinogenic metal salts. Cancer Res. 47:3815–3823 (1987).
27. Biedermann KA, Landolph JR. Role of valence state and solubility of chromium compounds on induction of cytotoxicity. mutagenesis and anchorage independence in diploid human fibroblasts. Cancer Res 50:7835–7842 (1990) .
28. Nishimura M, Umeda M. Induction of Chromosomal aberrations in cultured mammalian cells by nickel compounds. Mutat Res 68: 337–349 (1978).
29. Lavramendy ML, Popescu NC, DiPaolo JA. Induction by inorganic metal salts of sister chromatid exchanges and chromosome aberrations in human and Syrian hamster cell strains. Environ Mutagenesis 3:597–606 (1981).
30. Heck JD, Costa M. Review: *In vitro* assessment of the toxicity of metal compunds II. Mutagenesis. Biol Trace Elem Res 4: 319–330 (1982).
31. Reznikoff CA, Brankow DW, Heidelberger C. Establishment and Characterization of a Cloned Line of C3H Mouse Embryo Cells Sensitive to Post-Confluence Inhibition of Division. Cancer Res 33:3231–3238 (1973).
32. Reznikoff CA, Bertram JS, Brankow DW, Heidelberger C. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to post-confluence inhibition of cell division. Cancer Res 33:3239–3249 (1973).
33. Landolph JR,Heidelberger C. Chemical carcinogens produce mutations to ouabain resistance in transformable C3H/lOTl/2 Cl 8 mouse fibroblasts. Proc Natl Acad Sci USA 76:930–934 (1979).
34. Landolph JR. Chemical transformation in C3H/10Tl/2 Cl 8 mouse embryo fibroblasts: historical background, assessment of the transformation assay and evolution and optimization of the transformation assay protocol. Transformation assay of established cell lines: mechanisms and application. IARC Scientific Publications, Vol 146 (Kakanaga T, Yamasaki H, eds). Lyon:International Agency for Research on Cancer, 1985;185-193.
35. Landolph JR. Mechanisms of chemically induced multistep neoplastic transformation in C3H/lOTl/2 Cells. In: The Role of Chemicals and Radiation in the Etiology of Neoplasia: Carcinogenesis, Vol. 10 (Huberman E, Barr SH,eds). New York:Raven Press, 1985;211–223.
36. Landolph JR, Telfer N, Heidelberger C. Further evidence that ouabain-resistant variants induced by chemical carcinogens in transformable C3H/10T1/2 mouse embryo fibroblasts are mutants. Mutat Res 72:295–310 (1980).
37. Landolph JR, Bhatt RS, Telfer N, Heidelberger C. Comparison of adriamycin and ouabain-induced cytoxicity and inhibition of 86-Rb transport in wild-type and ouabain-resistant C3H/10T1/2 mouse fibroblasts. Cancer Res 40:4581–4588 (1980).
38. Landolph JR, Jones P A, Mutagenicity of 5-azacytidine and related nucleosides in C3H/10T1/2 and V79 cells. Cancer Res 42:817–823 (1982).
39. Landolph JR, Fournier REK. Microcell-mediated transfer of carcinogen-induced ouabain resistance from C3H/10T1/2 Cl 8 mouse fibroblasts to human cells. Mutat Res 107: 447–463 (1983).
40. Shibuya ML, MiuraT, Lillehaug JR, Farley RA, Landolph JR. Ouabain-resistant (Na,K)-ATPase activity in chemically induced ouabain-resistant C3H/10T1/2 cells. J Mol Toxicol 2: 75–98 (1989).
41. Milo GE, DiPaolo JA. Neoplastic transformation of human diploid cells *in vitro* after chemical carcinogen treatment. Nature(Lond) 275:130-132 (1978).
42. Greiner JW, Evans CA, DiPaolo JA. Carcinogen-induced anchorage-independent growth and *in vivo* lethality of human MRC-5 Cells. Carcinogenesis 2:359–362 (1981),
43. Silinskas KC, Kateley SA, Tower JE, Maher VM, McCormick J. Induction of anchorage-independent growth in human fibroblasts by propane sultone. Cancer Res 41:1620–1627 (1981).
44. Wetterhahn KE, People B, Kulesz-Martin M, Copeland ES. Meeting report; Workshop Report From the Division of Research Grants. National Institutes of Health. Metal Carcinogenesis — A Chemical Pathology Study Section Workshop, Cancer Res 52:4058-4063 (1992).
45. Ciccarelli RB, Wetterhan KE. Nickel-bound chromatin, nucleic acids, and nucleoproteins from kidney and liver of rats treated with nickel carbonate *in vivo*. Cancer Res 44:3892–3897 (1984).
46. Ciccarelli RB, Wetterhahn, KE. Nickel distribution and DNA lesions induced in rat tissue by the carcinogen nickel carbonate. Cancer Res 42:3544–3549 (1982).
47. Land H, Parada L, Weinberg RA. Cellular oncogenes and multistep

carcinogenesis. Science 222:771–776 (1983).
48. Weinberg RA. The action of oncogenes in the cytoplasm and nucleus. Science 230:770–776 (1985).
49. Bishop JM. The molecular genetics of cancer. Science 235: 305–311 (1987).
50. Klein G. The approaching era of the tumor suppressor genes. Science 238:1534–1546 (1987).
51. Horwitz JM, Yardell DW, Bark S-H, Canning S, Whyte P, Buchkoritch K, Harlow E, Dryja TP. Point mutational inactivation of the retinoblastoma anti-oncogene. Science 243:937–945 (1989).
52. Hollstein M, Sidransky D, Vogelstein B, Harris CC. P53 Mutations in human cancers. Science 253:4953 (1991).
53. Weissman BE Saxon PJ, Pasquale SR, Jones GR, Geise AG, Stanbridge EJ. Introduction of a normal human chromosome 11 into a Wilm's tumor cell line controls its tumorigenic expression. Science 236:175–180 (1987).
54. Klein CB, Conway K, Wang XW, Bhamra RK, Lin XH, Cohen MD, Annab L, Barrett JC, Costa M. Senescence of nickel-transformed cells by an X chromosome: possible epigenetic control. Science 251:796–799 (1991).
55. Tsapakos MJ, Hampton TH, Jennette KW. The carcinogen chromate induces DNA cross-links in rat liver and kidney. J Biol Chem 256:3623–3626 (1981).
56. Cupo DY, Wetterhahn KE. Binding of chromium to chromatin and DNA from liver and kidney of rats treated with sodium dichromate and chromium(III) chloride *in vivo*. Cancer Res 45:1146–1151 (1985).
57. Furst A, Schlander M, Sasmore DP. Tumorigenic activity of lead chromate. Cancer Res 36:1779–1783 (1976).
58. Lee T, Tanaka N, Lamb PW, Gilmer TM, Barrett JC. Induction of gene amplification by arsenic compounds. Science 241: 79–84 (1991).